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TITLE: THE ASSESSMENT OF THE TECHNICAL REQUIREMENTS OF A CLOSED BLOOD HANDLING SYSTEM USING A TRI-ENZYMES FOR A HIV ASSAY - SBIR 89.I (A89-072)

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PREPARED FOR: U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
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The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

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U.S. DEPARTMENT OF DEFENSE

SMALL BUSINESS INNOVATION RESEARCH PROGRAM
PHASE 1 – FY 1989
PROJECT SUMMARY

Topic No. A89-072 Military Department/Agency Army

Name and Address of Proposing Small Business Firm

KEA Industries
2080 Experiment Farm Road
Troy, Ohio 45373

Name and Title of Principal Investigator

Albert E. Katzer, Director, Engineering

Proposal Title

The Assessment of the Technical Requirements of a Closed Blood Handling System using a Tri-Enzyme Enzymes for a HIV Assay.

Technical Abstract (Limit your abstract to 200 words with no classified or proprietary information/data.)

This funded Research Effort assessed the technical requirements to develop a closed blood handling device and testing vehicle for Human Immunodeficiency Virus (HIV) utilizing ELISA protocol as well as the encapsulation of the multi-enzymes within the closed system. It was a design/modeling program to modify an existing single cavity cuvett developed for Non A, Non B Hepatitis to the multi/ process system required by the ELISA protocol. The Auto-cuvette proto-type designed within this Research Project is a seven stage automated blood testing vehicle using Wafer Technology to encapsulate the multi-enzymes required by the ELISA protocal. The design allows complete safety from HIV contamination; (1) The coated cuvet cell is received from the supplier with a sealing foil in place for operator protection. This foil will be pierced at the first stage, (2) All offal from both wash stations is carried into a disposable reservoir, and (3) The coated "cuvet" cell used for absorbance measurement is automatically sealed before removal from the Auto-cuvette.

Anticipated Benefits/Potential Commercial Applications of the Research or Development

Allow a rapid, safe, and economical "Pre-Donor" testing of blood for HIV and other contagious diseases in the hospital, the field, and Third world Countries.

List a maximum of 8 Key Words that describe the Project.

Closed Assay Device for Testing Blood for Diseases

Table of Contents

Table of Contents-Cont.

SECTION I. **INTRODUCTION**

A. The purpose of this Research Effort was to assess the technical requirements needed for the development of a closed blood handling device and testing vehicle for a Human Immunodeficiency Virus (HIV) assay utilizing ELISA multi-enzyme and/or Recombinant DNA methods. The original evaluation was to consider both the ELISA multi-enzyme and/or Recombinant DNA methods and a competitive binding immunoassay incorporating a separation free technology as used in a classical antigen reaction. Due to the shortness of the research time this project was limited to the **ELISA** multi-chamber system.

B. The detection of the HIV (Human Immunodeficiency Virus) antibody or antigen has been a time consuming and complex process because of the MULTI-STEP nature and individual handling required due to the antigen-antibody reactions technique developed in the existing ELISA and Recombinant DNA methods.

C. The proposed research was oriented to investigate what needed to be identified in the evaluation, adaption, and modification of various cuvette designs,

including the design developed for Micro Diagnostics for use on the Non A, Non B hepatitis assay, to handle immunodiagnostic and/or DNA probe assays for HIV analytes. Closed blood handling devices and testing vehicles were evaluated considering the ELISA multi-enzyme and/or Recombinant DNA methods as well as the safety of the laboratory technicians.

D. The original "Pre-Donor Screen system" including the single stage cuvette was designed for the detection of Non A, Non B Hepatitis utilizing the surrogate ALT (Alanine aminotransferase) enzyme methodology.

E. The technological innovation within Micro Diagnostic's System was the System's ability to measure analytes, spectophotometrically, at extremely low concentration levels in a kinetic mode while the System was being operated in the field (internally battery powered or power via external source). Also there is a distinct possibility that this system because of its "field orientation and low cost" will have a Third World application for HIV screening. There is a need to further refine the cuvette (disposable blood handling device for the Pre-Donor Screening Device). In addition, the AIDS crisis has

heightened the awareness by individuals who handle and process blood products and by-products of the importance of taking the necessary handling precautions. Hence the need for an inexpensive, safe closed blood handling device for HIV testing.

F. Phase One will assess the technical requirements needed to develop a closed blood handling device and testing vehicle for an HIV (Human Immunodeficienvy Virus) assay to be used in equipment similar to Micro Diagnostic's for HIV adaption of its Pre-Donor Screening Device for Non A Non B Hepatitis. It will be the design/modeling program to modify an existing single cavity cuvette to the possible multi/process system which is required by the existing ELISA and Recombinant DNA methods. Micro Diagnostic's "Pre-Donor Screen" type instrumentation with the proper software modifications to accommodate immunodiagnostic measurements and data reduction will be used. Several iterations of this process is anticipated with the end result being an efficient, more refined cuvette design for HIV analytes.

G. Structurally and biochemically, HIV (AIDS) belongs to the retrovirus family, a unique subgroup of virus found not only in humans but also in many animals, from

reptiles to primates. Like other viruses, retroviruses do not always cause disease in their hosts. Infection occurs when a virus attaches itself to highly specific receptors on the cell's protein coat and thus penetrates the susceptible cell. Unfortunately the detection of the HIV antibody or antigen has been a time consuming and complex process because of the nature of the antigen-antibody reactions ie ELISA methods.

H. KEA Industries will address the design of the cuvette modification to accommodate the limiting factors found in various ELISA and Recombinant DNA methodologies. These limiting factors were evaluated in terms of operation environment, temperature, sample size consistency, incubation requirements, and material interface compatibility, etc. In addition to the more standard methodologies being evaluated, new approaches will were reviewed. For example Dr. Bruce E Kemp's (University of Melbourne) approach using a single stage analysis with synthetic peptide antigen conjugated with whole human blood may prove to be acceptable to the cuvette design, etc. It appears that the synthetic peptide antigen may easily be packaged within the closed system similar to the one developed and should be reviewed in a future program.

I. In Phase Two, production dies will be designed and built to allow the fabrication of cuvette assemblies for further laboratory analysis. Further pertubations necessarily introduced into the assay procedures upon which the cuvette is to be designed around will be further evaluated. A close working relationship will be required between Walter Reed Army Institute of Research and KEA Industries for pre-product testing of the system. The end result of Phase Two would be the finalization of a cuvette pre-production design which addresses the needs of the user (safety in blood handling, convenience and low cost) and limited production of the Auto-cuvette. During Phase Two instrumentation will be modified for use within the system.

SECTION II. CONCLUSION

A. A proto-type design of a seven stage **Auto-cuvette System** has been completed and the operational stations detailed. The completed design proposes a single section, seven stage cuvette which, although it uses Organon's diagnostic kit as a model, the combination of reagents, dispensing times and amounts, as well as temperature constraints are similar to other **ELISA** kits already on the market.

1. The proto-type design presented in **Monthly Technical Report No. 5** was a six stage system. The seventh stage was added so that the "cuvet" containing the test sample would be capped to protect the technician during the final sample analysis. This would be an automatic procedure at the final stage.

B. The use of **wafer technology** in the **Auto-cuvette System** offers extreme tight control of the diagnostic procedures and allows excellent repeatability of the **ELISA** methodology protocol of both the sample and controls.

C. The proposed seven stage auto-cuvette **ELISA** protocol offers the following:

1. The **Auto-cuvette** design developed in this project is for use with the **ELISA** protocol. Organon Teknika's system is a ten step system taking approximately **3.5** to 5 hours to complete the test to absorbance measurement. The **Auto-cuvette** design will reduce this time to approximately **2.2** to **2.5** hours from load to absorbance measurement.

2. The **Auto-cuvette** design with its related hardware will be an automatic process allowing the technician to carry out other duties during processing time.

3. The **Auto-cuvette** design allows complete safety from **HIV** contamination because of its design:

a. The **coated** "cuvet" cell will be received from the supplier with a sealing foil in place for operator protection. This foil will be pierced at the first stage of the procedure.

b. All offal from both wash stations is carried into a disposable reservoir attached to the **auto-cuvette.** When the reservoir is removed from the cuvette for disposal, a sealing cap will automatically be in place.

c. The coated "cuvet" cell used for absorbance measurement is automatically sealed before removal from the **auto-cuvette** for absorbance measurement.

d. The basic **auto-cuvette** can be disassembled, sterilized, and reloaded before the next test. The design is such that the basic unit has no or limited contact with the virus.

SECTION III. **RECOMMENDATIONS**

A. The evaluation of the technical requirements of a closed blood handling device and testing vehicle for **HIV** assay utilizing **ELISA** methodology has been successfully completed and is outlined in the DISCUSSION, Abstract A, and Abstract B of this report.

B. Due to success of **PHASE** I It is recommended that a **PHASE** II be approved to cover the following objectives:

1. An interactive program is required between Walter Reed Army Institute of Research and KEA Industries to run tests and evaluate the success of the **Auto-cuvette** system.

2. The **Auto-cuvette** design will be modified to allow two control samples to be processed at the same time as the patient's sample.

3. A. **spectrophotometer** will be modified to accept the capped coated "cuvet" cell for

absorbance measurement.

4. An indexing and timing mechanism will be designed and built to position the Auto-cuvette.

5. The basic Auto-cuvette developed under Phase One will be modified to process two controls with each sample.

SECTION IV. DISCUSSION

A. On May 3, 1989 the U.S. Army Medical Research Acquisition Activity approved a Phase project with KEA Industries to assess the technical requirements needed for the development of a closed blood handling device and testing vehicle for a Human Immunodeficiency Virus (HIV) assay utilizing ELISA multi-enzyme and/or Recombinant methods.

B. The proposed research was oriented to investigate what needs to be identified in the adaption of a single stage cuvette (originally designed by KEA Industries for Micro Diagnostics, Inc. "Pre-Donor Screen System" for Non A, Non B Hepatitis)to handle immunodiagnostic and/or DNA Probe assays for HIV analytes. The original "Pre-Donor Screen system" was designed for the detection of Non A, Non B Hepatitis utilizing the surrogate ALT (Alanine aminotransferase) enzyme methodology. The technological innovation within this system was the system's ability to measure reactions, spectophotometrically, at extremely low concentration levels in a kinetic mode while the system was

being operated in the field (internally battery powered or power via external source).

C. Given the ability of this system to measure analytes at extremely low levels of concentration and the distinct possibility that this system because of its "field orientation and low cost" of having Third World application for HIV screening, the need to further refine the cuvette (disposable blood handling device for the Pre-Donor Screening Device) is evident. In addition, the AIDS crisis has heightened the awareness by individuals who handle and process blood products and by-products of the importance of taking the necessary handling precautions. Hence the need for an inexpensive, closed blood handling device for HIV testing.

D. In Phase II, production dies would be designed and built to allow the fabrication of cuvette assemblies for further laboratory analysis. Further pertubations necessarily introduced into the assay procedures upon which the cuvette is to be designed around would be further evaluated. The end result of Phase II would be the finalization of a cuvette pre-production design which addresses the needs of the user (safety).

E. Early in September, 1989 a brief discussion was held with Professor Don Shive of Muhlenber College on his experiences with a closed cuvette system. Of particular interest was the dispersion and mixing of ALT with the distilled water, the injection of the blood sample and the decay time of the reaction.

F. On 13 September 1989 a Sonics & Materials' EM-1500/1098 microprocessor controlled ultrasonic plastics assembly system was ordered to fabricate the various sample cuvettes to be used in this development project. This unit will also be used in the processing of the wafers which will carry the reagents. Equipment was received on 4 December 1989 and is installed.

G. A proto-type design of the six stage cuvette assembly has been completed and is presently being detailed. A sketch of this proto-type was presented in Monthly Technical Report No. 4.

H. The preliminary cuvette design presented was developed for consideration for use in the ELISA system. **Organon Teknika's** system is a ten step system taking approximately 3.5 to 5 hrs. to complete the test to

absorbance measurement. As outlined in Report No. 3, in the development of the multiple chamber cuvette system ten steps are required to complete the HIV-1 Protocol. Seven positions are required to accomplish this in the new design. A summary of these steps is outlined on Page 25 of this report.

G. The HIV-1 Protocol proposed in Monthly Technical Report No. 4 was reviewed by RAK Associates and their recommendations are carried in complete context in Appendix C of this report.

H. The following items which were presented in RAK Associates reports have been addressed and modifications were made to the proposed HIV Protocol. The new HIV Protocol is outlined Appendix A of this report.

1. The HIV-1 antigen will be coated on the inside of the cuvette "cell". The distance on the inside of the cuvette "cell" is .35 inches, allowing .040 inches for the two walls of the cell. Spectrophotometer light path must be held to one centimeter or .39 inches

2. The coated cuvette cell

will be received from the supplier with a sealing foil in place for operator protection. This foil will be pierced in the first operation. (See Page 27 in Appendix A and Page 39 in Appendix B).

3. At each washing stage special protection is designed into the cuvette assembly to limit contamination. Although the proto-type under design will handle only one sample, the same design will protect production units from cross cell contamination.

4. In Report No. 3, Appendix C, RAK Associates referred to the prior mixing and shelf life of the various units used in the HIV Protocol.

a. The test procedure outlined in Appendix A of this report outlines extensive use of wafer encapsulation of the various compounds used in this protocol. An extensive future investigation will be required to develop the shelf life of these pre-assembled cuvettes.

5. As outlined in RAK Associates Report No. 3, Appendix C, production applications of this

Protocol will require extensive use of labels (Bar Codes).

6. Report No. 2 presented in Appendix C by RAK Associates reviewed the preliminary protocol outlined in the early reports. This information was used to correct the proposed HIV testing Protocol. (See Appendix A of this report).

I. Encapsulation of **DILSIM, WASH SOLUTION,** reconstituted **EnzAbody, ABTS** substrate, and the **STOP** solution in the wafer assemblies is included in the new proposed HIV Protocol.

1. Additional material development is required for the wafers to be used with the STOP solution of the cuvette assembly. The materials developed for use in other areas of the cuvette assembly are not compatable with **Sodium Floride.** (This must be reviewed in Phase II)

J. The design of the proto-type cuvette assembly considered only one sample to be processed at a time. (A production protocol system for HIV will process three samples in parallel; 1. Subject's sample, 2. A negative sample, and 3. A positive sample.

The following proto-type HIV Protocol is proposed in this design. (See Page 25 and Appendix A of this report)

1. DISIM, WASH SOLUTION, reconstitutaed EnzAbody, the prepared ABTS, and the STOP solution will be ultra-sonic encapsulated in various wafer configuration and installed in the required sections of the cuvette assembly.

2. In position one, stage one of the cuvette assembly approx. 5ul of the subject's serum or plasma will be collected in a standard capillary tube. This capillary tube is inserted into the upper plunger of position No. 1. An activating mechanism forces the upper plunger down breaking the Dilsim wafer assembly and piercing the foil cover on the cuvette cell and inserting the Dilsim and the subject's sample into the HIV-1 coated cuvette cell. A low frequency vibrator assists in the mixing.

3. The cuvette coated cell will remain in position one, and will be heated by an Infared system to 37 degrees Centigrade for 90 to 100 minutes.

4. The cuvette coated cell will be in position 2 for the washing stage. This station is so designed that exposure of the system to the washing solution will have two to three times the effectiveness of a normal HIV protocol wash. The wash solution had been previously packaged in a wafer assembly and placed in position 2 of the cuvette assembly. Its volume is designed to give an effective wash volume of six times.

The activating mechanism forces the upper plunger down breaking the Wash wafer assembly. The plunger continues into the cuvette causing all surfaces to be washed under pressure. The wash solution is forced into a reservoir within the assembly.

5. The cuvette coated cell will be in position 3, stage 1 for adding the reconstituted EuzAbody to the cell. The activating mechanism forces the upper plunger down piercing the reconstituted EnzAbody wafer assembly and inserting the material into the HIV-1 coated cell. A low frequency vibrator assists in the mixing.

6. The coated cuvette cell with

the added EnzAbody must remain in position to allow for incubation of the samples. Using a directed Infrared heating system built into the activating mechanism the sample is incubated for 30 to 35 minutes at 37 degrees Centigrade.

7. The coated cell is moved to position 4, stage 1 where it is washed with the same type of mechanism as discussed in item 4 above.

8. The cuvette coated cell will be in position 5, stage 1 for adding the ABTS solution to the cell. The activating mechanism forces the upper plunger down breaking the prepared ABTS wafer assembly and inserting the material into the HIV coated cell. A low frequency vibrator assists in the mixing.

9. The coated cuvette cell with the ABTS solution must remain in position 5 to allow time for its reaction. No heating is used at this stage, only room temperature (20 to 25 degrees Centigrade). The sample is incubated for 10 to 12 minutes.

10. The cuvette coated cell will

be in position 6 for the addition of the STOP solution. The activating mechanism forces the upper plunger dow piercing the prepared STOP Solution wafer assembly and forcing the material into the HIV-1 coated cuvette cell. A low frequency vibrator assists in the mixing.

11. In position 7 the processed cell is removed from the cuvette assembly for absorbance reading. A cap built into the assembly closes the cell during removal to protect the operator. The absorbance is read at 405nm or 690nm.

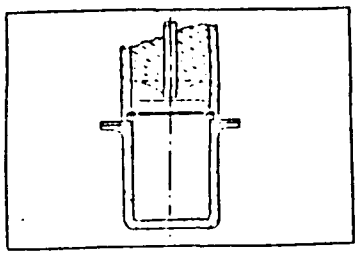
K. The time elements on the various stages of the **ELISA** test procedure has to be taken into consideration in the new **Auto-cuvette** design. At this stage all development was concentrated on the seven chanber system.

L. The design presented in this report will require a modification of the spectrophotometer used to read the absorbance. This design does not allow for a standard comparison as allowed in a normal 12 well **HIV** Microelisa System. In Phase II this comparison must be included in a production version.

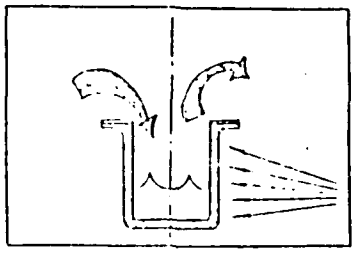
M. At this stage of the development all areas of investigation are complete and design modifications have been considered except the addition of the control stations. This will be added in the early design stages of Phase II.

B. The time elements on the various stages of the ELISA test procedure have been taken into consideration in the new cuvette design. The feasibility study has established a need for a seven stage system to allow for automation of the proposed test procedure.

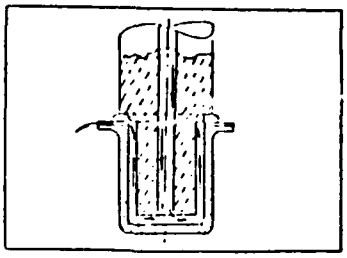
C. Appendix B in this report give an outline on the design characteristics built into the Auto-cuvette. Appendix A explains the protocal steps required in the ELISA test procedure adapted in this study.



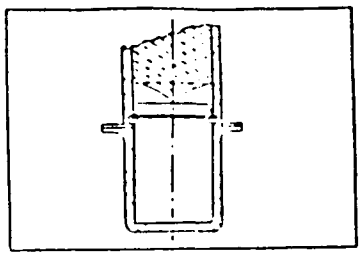
1 Add 3 µl of samples and controls plus 225 µl of DILSIM into the HIV coated microplate wells with a diluter/dispenser. Mix contents by tapping plate.



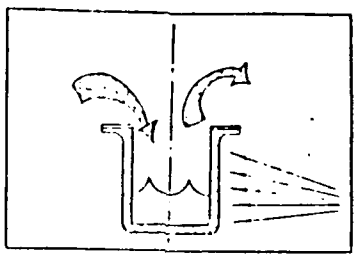
2 incubate for 90 to 100 minutes at 37°C.



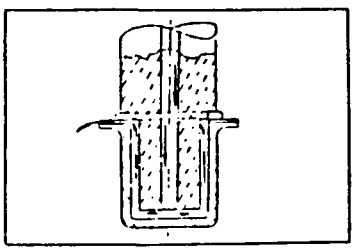
3 Aspirate and wash each well four times with diluted Wash Solution.



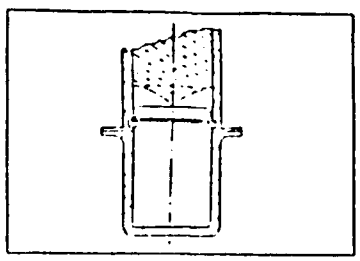
4 Add 150 µl of reconstituted EnzAbody into each well. Mix by tapping.



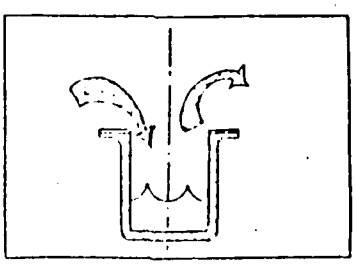
5 incubate for 30 to 35 minutes at 37°C.



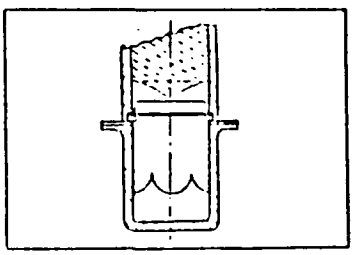
6 Aspirate and wash each well four times with diluted Wash Solution.



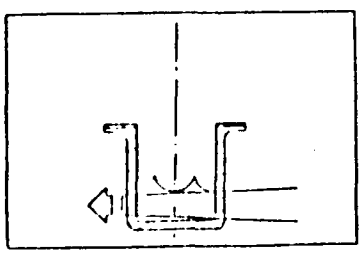
7 Add 150 µl of prepared ABTS Substrate into each well. Do not mix.



8 Incubate for 10 to 12 minutes at room temperature (20 - 25°C). Do not cover with plate sealer.



9 Add 150 µl of Stop Solution into each well. Mix by tapping.

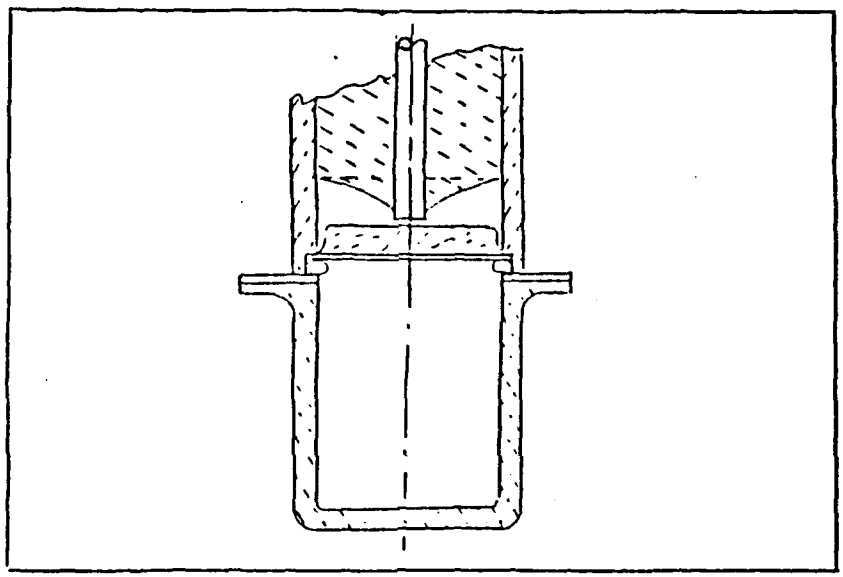


10 Read absorbance at 405 nm or 690 nm.

PROPOSED PROTOCOL TEST
PROCEDURE - HIV
(See Appendix A)

Appendix A

EXAMPLE #1.
Position 1, Stage 1



ADDING PATIENT'S SAMPLE AND DILSIM TO COATED CUVETTE

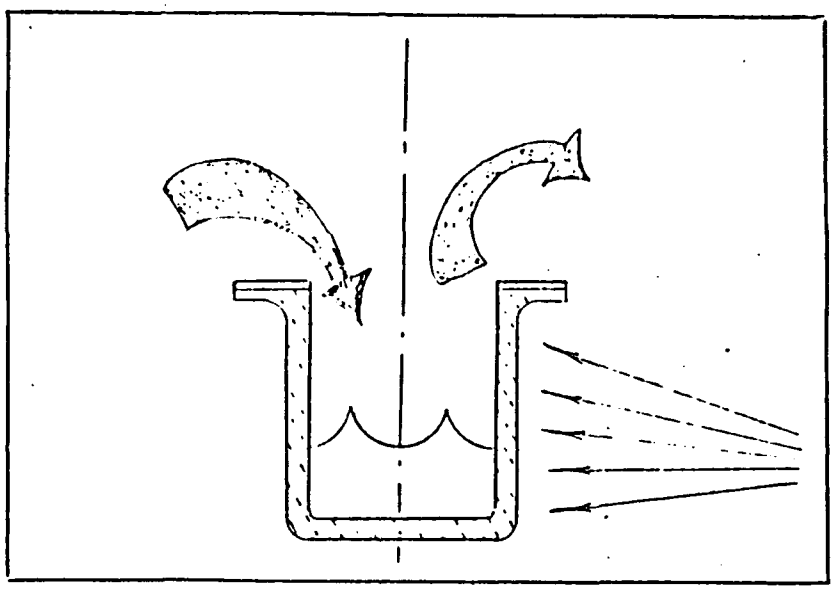
Approximately 5ul of the subject's serum or plasma collected in a standard capillary tube is placed into the upper plunger of position No. 1. The cover cap is snapped into place.

The Dilsim prepared from the Diluent mixture had been previously encapsulated in the wafer assembly and installed in station one (see the above sketch).

The activating mechanism forces the upper plunger down breaking the Dilsim wafer assembly and the foil cover on the cuvette and inserting the subject's sample into the HIV coated cuvette.

A low frequency vibrator which is part of the mechanism assists in the mixing.

EXAMPLE #2.
Position 1, Stage 2

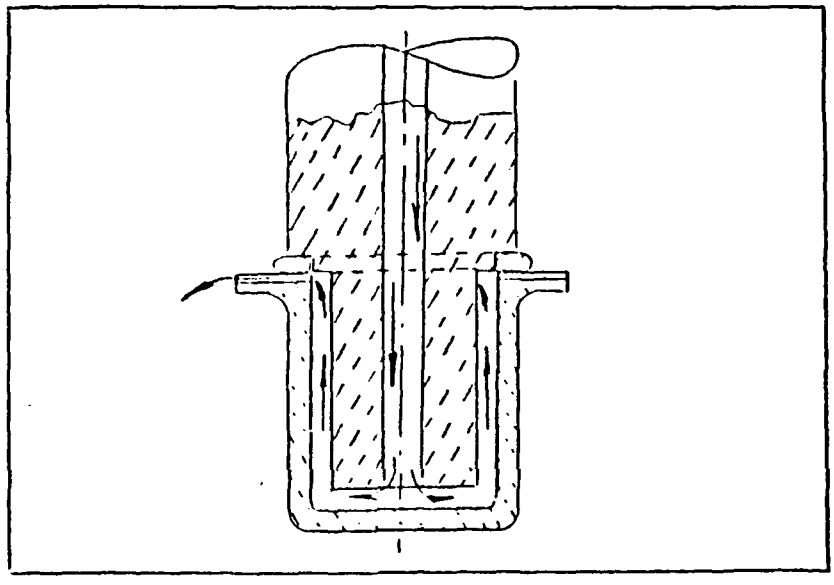


FIRST INCUBATION OF TEST SAMPLE

Test sample has not been moved from position one.

Using a directed Infared heating system built into activating mechanism the sample is incubated for 90 to 100 minutes at 37 degrees Centigrade.

EXAMPLE #3.
Position 2, Stage 1



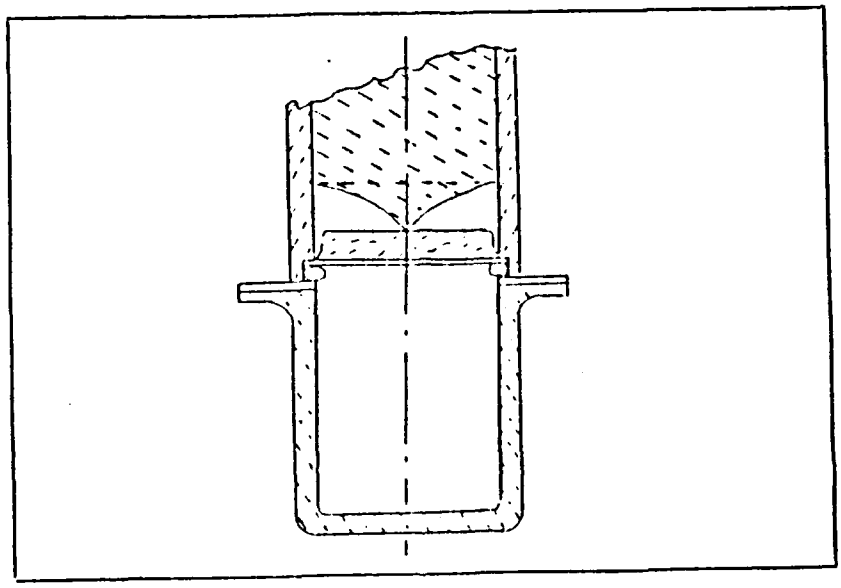
WASH STATION

The Wash Solution had been previously been mixed at a 1:50 with distilled water. The Wash Concentrate contains a 2.5% surfactant.

The wash solution had been previously packaged in a wafer assembly and placed in position 2 of the cuvette assembly. Its volume is design so as to give an effective wash volume of six times.

The activating mechanism forces the upper plunger down breaking the Wash wafer assembly. The plunger continues into the cuvette causing all surfaces to be washed under pressure. The wash solution is forced into a reservoir within the mechanism.

Example 4
Position 3, Stage 1



ADDING EnzAbody TO THE COATED CUVETTE

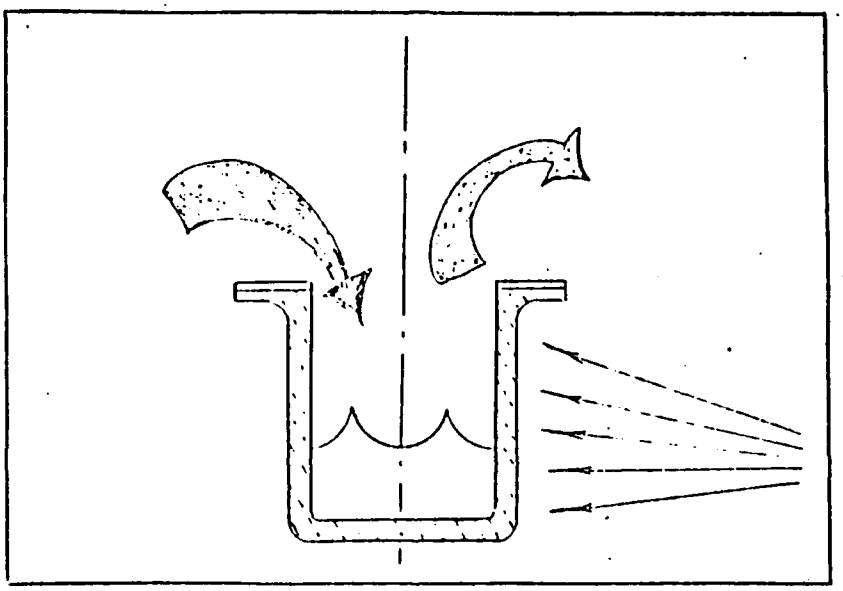
50ul of prepared diluent had previously been mixed with 1 vial of EnzAbody had been previously been mixed to form 150ul of reconstituted EnzAbody and had been encapsulated in a wafer assembly.

This EnzAbody wafer assembly had previously been installed in position 3 of the cuvette assembly.

The activating mechanism forces the upper plunger down breading the reconstituted EnzAbody wafer assembly and inserting the material into the HIV coated cuvette.

A low frequency vibrator which is part of the mechanism assists in the mixing.

EXAMPLE #5.
Position 3, Stage 2

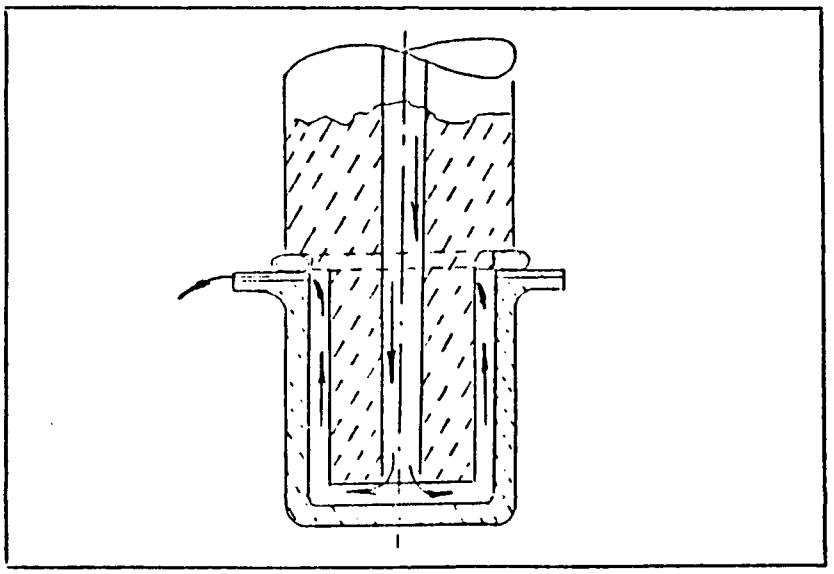


SECOND INCUBATION OF TEST SAMPLE

Test sample has not been moved from position three.

Using a directed Infared heating system built into activating mechanism the sample is incubated for 30 to 35 minutes at 37 degrees Centigrade.

EXAMPLE #6.
Position 4, Stage 1



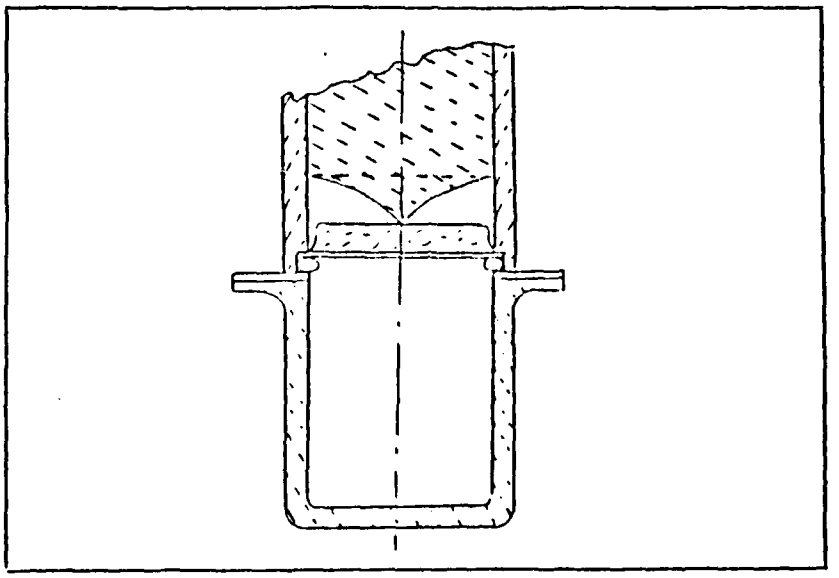
WASH STATION

The Wash Solution had been previously been mixed at a 1:50 with distilled water. The Wash Concentrate contains a 2.5% surfactant.

The wash solution had been previously packaged in a wafer assembly and placed in position 4 of the cuvette assembly. Its volume is design so as to give an effective wash volume of six times.

The activating mechanism forces the upper plunger down breaking the Wash wafer assembly. The plunger continues into the cuvette causing all surfaces to be washed under pressure. The wash solution is forced into a reservoir within the mechanism.

Example 7
Position 5, Stage 1



ADDING PREPARED ABTS TO THE COATED CUVETTE

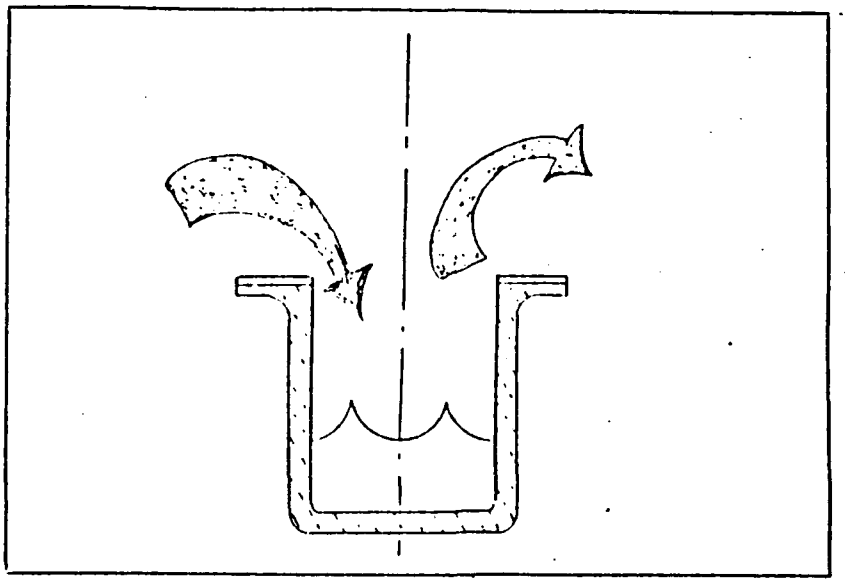
75ul of prepared ABTS diluent had previously been mixed with 75ul of ABTS Substrate had been previously been mixed to form 150ul of prepared ABTS and had been encapsulated in a wafer assembly.

This ABTS wafer assembly had previously been installed in position 5 of the cuvette assembly.

The activating mechanism forces the upper plunger down breaking the prepared ABTS wafer assembly and inserting the material into the HIV coated cuvette.

A low frequency vibrator which is part of the mechanism assists in the mixing.

EXAMPLE #8.
Position 5, Stage 2

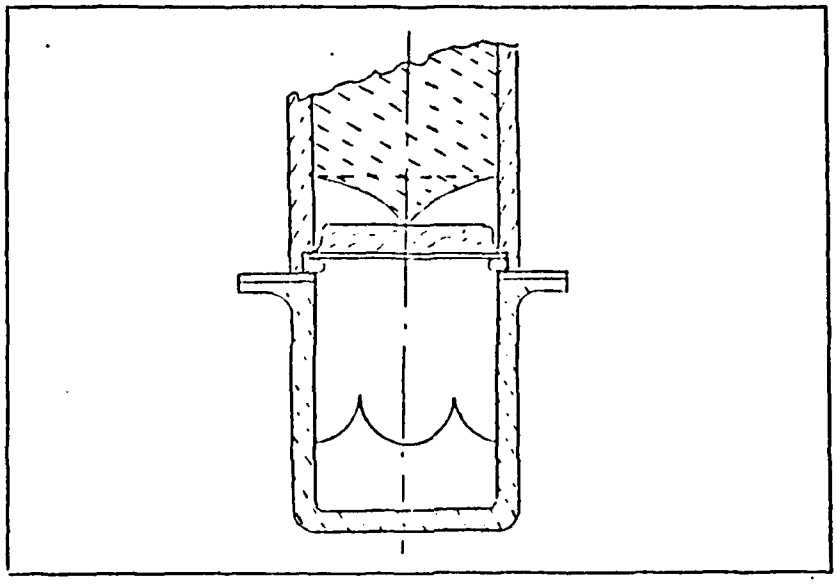


THIRD INCUBATION OF TEST SAMPLE

Test sample has not been moved from position five.

No heating is used at this stage, only room temperature (20 to 25 degrees Centrigrade. The sample is incubated for 10 to 12 minutes.

Example 9
Position 6, Stage 1



ADDING STOP SOLUTION TO THE COATED CUVETTE

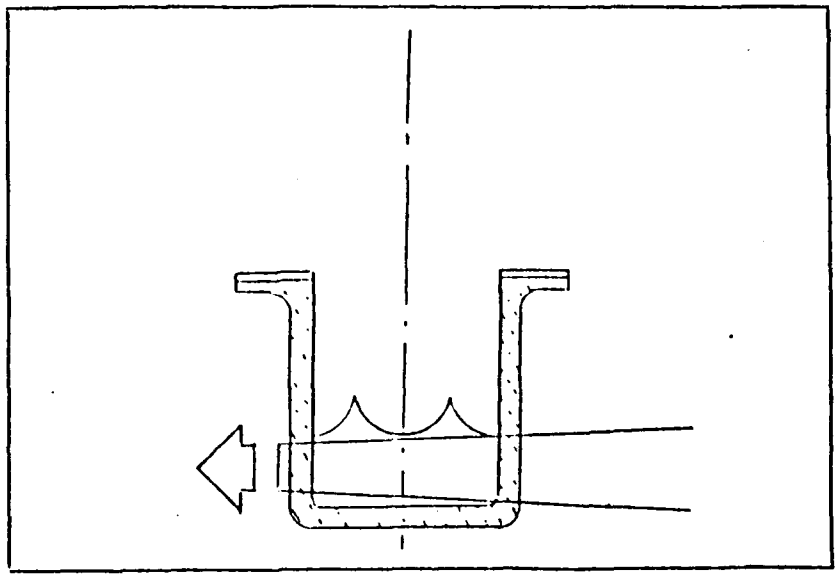
150ul of prepared STOP Solution had previously been mixed with a soltion of 0.28% Sodium Floride and had been encapsulated in a wafer assembly.

This STOP Solution wafer assembly had previously been installed in position 6 of the cuvette assembly.

The activating mechanism forces the upper plunger down breaking the prepared STOP Solution wafer assembly and inserting the material into the HIV coated cuvette.

A low frequency vibrator which is part of the mechanism assists in the mixing.

EXAMPLE #10.
Position 6, Stage 2



READ ABSORBANCE

The cuvette must be read within two hours after STOP Solution is added.

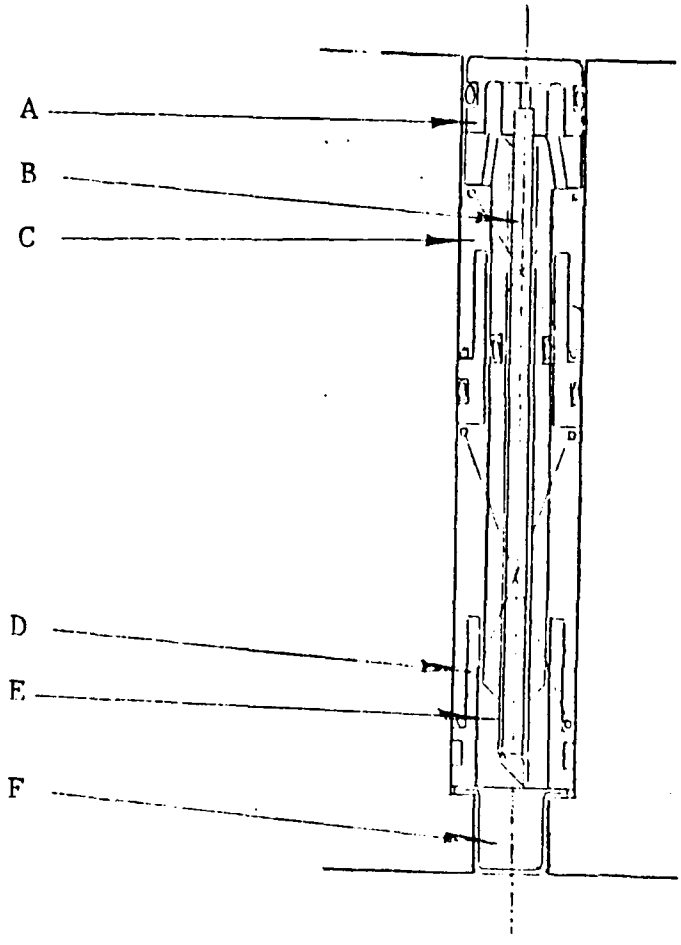
Read absorbance at 405nm or 690 nm.

Appendix B

INTERNAL MECHANISMS

A. On the following pages, 39 through 42, a brief description is given on the three main mechanisms and the wafer assembly of the Auto-cuvette and how these mechanisms function. The design technologies presented here are **unique** and are CONFIDENTIAL.

B. The seventh position is not shown. It is a simple plunger which caps the processed "cuvet" coated cell prior to being removed from the Auto-cuvette and placed into the modified spectrophotometer for absorbance measurement.



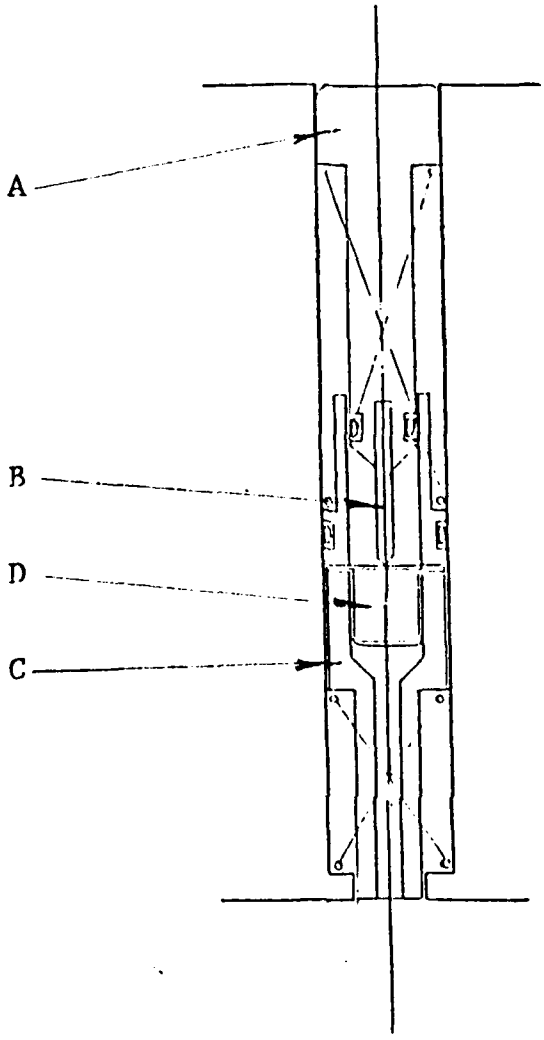
ADDING PATIENT'S SAMPLE AND DILSIM TO COATED CUVETTE
(Position 1, Stage 1)

A. This mechanism is responsible for piercing the wafer and coated "cuvet" seal and ejecting the sample into the "cuvet.

1. The subject's serum or plasma collected in a standard capillary tube is placed into cap "A" and the cap and Capillary tube is inserted into the plunger ass'y as shown at "B".

2. The activating mechanism forces the plunger down causing the stylus "E" to pierce the wafter assembly and the foil cover of the coated "cuvet. The DILSIM mixture is forced into the coated "cuvet"and the upper assembly is bottomed on "D".

3. The activating mechanism continues down creating a pressure build up in "C" forcing the subject's sample into the coated "cuvet".

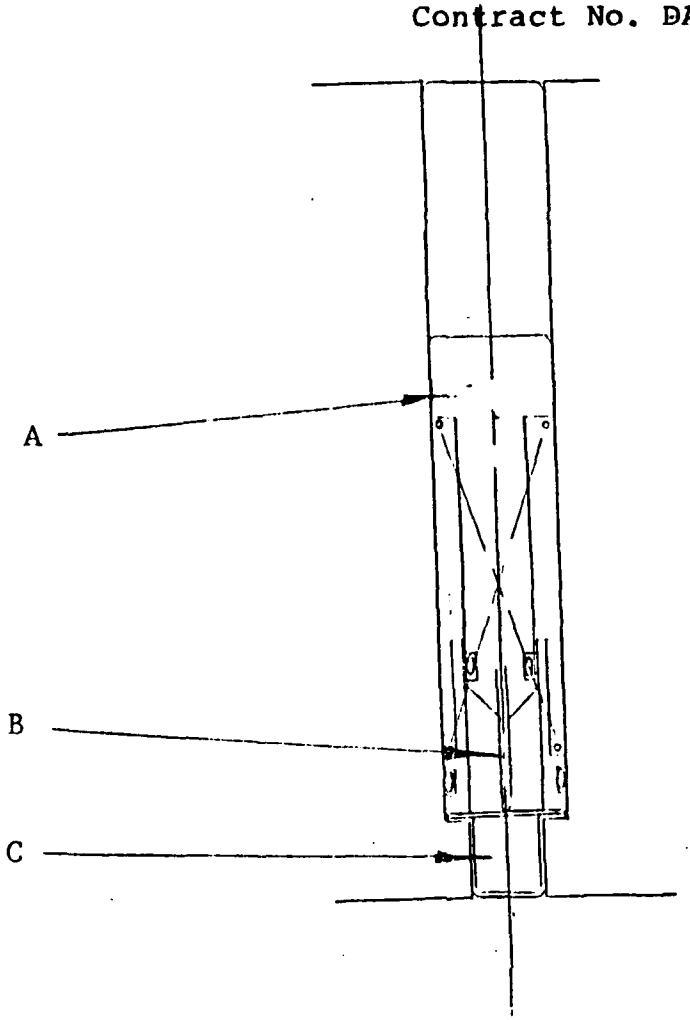


WASH STATION
(Position 2, Stagel)
(Position 4, Stage 1)

A. The wash solution had been previously packaged in a wafer assembly. The activating mechanism forces the plunger into the coated "cuvet" causing all surfaces to be washed under pressure with the solution to be forced into a reservoir within the Auto-cuvette.

1. The activating mechanism forces plunger "C" down into the coated "cuvet"until it bottoms out on the lower spring.

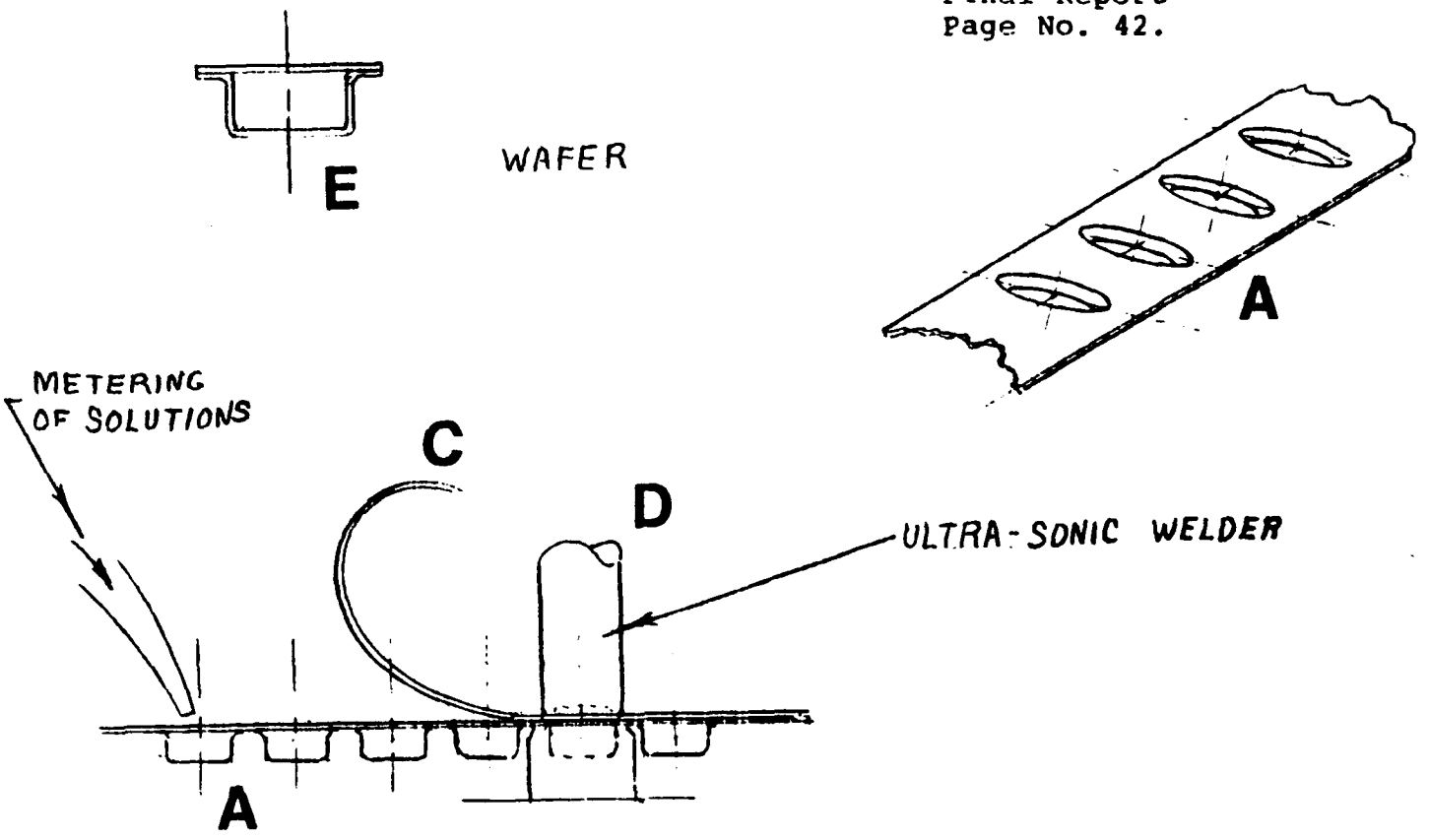
2. The upper plunger "A" continues down causing stylus "B" to pierce wafer assembly "D". The upper plunger continues down forcing Wash Solution from "D" into the coated "cuvet".



ADDING Enzbody TO THE COATED "CUVET"
(Position 3, Stage 1)
ADDING PREPARED ABTS TO COATED "CUVET"
(Position 5, Stage 1)
ADDING STOP SOLUTION TO THE COATED "CUVET"
(Position 6, Stage 1)

A. Plunger assemblies allows the insertion of various solutions into the coated "cuvet" at the required stations of the ELISA protocal.

1. The activating mechanism forces the plunger "A" down allowing stylus "B" to pierce the previously prepared Wafer assembly "C". Plunger "A" continues down forcing the required solutions into the coated "cuvet".



WAFER ASSEMBLY
(Solution Carrier)

A. The Auto-Cuvette development for the ELISA protocal requires the use of wafer assemblies carrying the correctly metered solutions required at the various stages of the protocal. There are four stages to this assembly; (1) Forming of the resovoir, (2) The metering of the solutions, (3) Ultra-sonic welding of the top to the resovoir, and (4) punching of the assembly out of the tape.

1. Resovoirs will be formed in a strip tape, "A".

2. The solution will be metered into the resovoir "B".

3. The top tape "C" will be ultra-sonic welded to the filled resovoir "D".

4. The wafer assembly "E" will be punched from the tape for installation in the Auto-cuvette.

Appendix C

SUBJECT: Report No. 1 from RAK Associates, 4 January 1990

RE: KEA Industries's HIV Cuvette Design

A. **Current Design modification per Report #4**

The HIV-1 antigen would have to be coated on the inside of the cell ie. referencing your drawing the section that is .35 x .25 inches. The coating would be only on one side, the other side would be non coated. The long side of the cuvette container connecting to each of these .25 x .35 needs to be adjusted(in my opinion) so that it is one centimeter in length (that is the outside distance between both .25 x .35 inch sides). Keep in mind the light path in almost all spectrophotometers are designed for absorbance measurements for 1 cm light paths. Therefore I would recommend that an adjustment be made to the design here.

The design of the spectrophotometer for this adaption would be for the light path to pass through the cuvette section hortizontally. The disadvantage in this design as I view it would be the limitation on the number of samples that can be run. This design would only allow one

sample per run...not a very marketable concept.

B. **Another adaption of Current Design modification per Report # 4**

Utilization of the current cuvette exterior but modified to accept the individual Microtiter wells provided in Organon's HIV kit would allow the simulataneous analysis of the sample, positive and negative control, run in parallel. (In addition the establishment of the standard curve required in other ELISA based immunoassay protocols could be accommodated althouugh for the purpose of this project the standard curve preparation is not required... Organon's protocol for its HIV-1 Diagnostic Kit only requires positive and negative controls).

The design of the spectrophotometer's measurement section would be different that above. In this section the light beam would be passing from the bottom vertically through the cuvette, exiting at the top.

The adantage of this type of design is that it takes into account Organon Teknika's HIV-1 Microelisa

diagnostic kit design.

additional comments:

(1) Utilizing adaption B would indicate that the design of the washing sections needs to be revaluated. The following considerations need to be taken into account.

a. Each microtiter well must be washed such that contamination from one well to the other is non existant.

b. A puncturing device such as a disposable pipet tip in which the foil affixed to the cuvette tip is punctured and the patient sample is injected could be used.

c. I believe with this adaption (B), your wafer design could be applicable. Over each of the microtiter wells in one or more of the container stages, the wafer could contain the conjugate, etc. and be punctured when required. This approach would allow for the processing in parallel, of more than one sample. This could also simplify the design of the plastic container in that the reagents

(chemicals) that need to be separated because either of expense (expensive reagents indicates a need for a little waste as possible) or a specific volume of reagent needs to be dispensed into the microtiter well as outlined in the protocol...the ability of consistently dispensing the same amount of reagent into the microtiter well need to be taken into account.

SUBJECT: Report No. 2 from RAK Associates, 7 January 1990

RE: KEA Industries Cuvette Design Incorporating HIV-1 Organon Teknika Protocol.

A. **First Draft Protocol**

Step 1: Pipet patient's sample into each hole(position No. 1).

The customer would utilize their own pipetting device to dispense(pipet) approximately 5ul of serum or plasma into the cuvette. I would envision that four holes on the top of the cuvette assembly, located at one end, would be adequate. The amount of serum or plasma is not that critical here because the dispensing of 5ul would be accomplished in step 2.

Step 2: Insert Cap and depress.

I envision a small flat looking cap with four protrusion of a specific length coming out of the bottom of the cap. These protrusions would be inserted into

the four holes in one step and pressed firmly into place. It would be important that the cap be of a stiff material because the length of the protrusions regulates the amount of serum or plasma pipetted into the four holes(step 1) finally dispensed into the micro titer wells(MTW). At this point the cuvette assembly is completely sealed. The cap that has been inserted could not easily be removed.

Step 3: Slide Bar across top of container until it locks with MTW on bottom. Upon completion invert "cuvette" assembly two or three times and place on work area with "bar" side facing upward.

This step does nothing more than position the Micro Titer Well for processing utilizing the remaining stages of the cuvette assembly. The bar by depressing it in various locations acts as a mechanical lever and an indicator to the operator as to where in the protocol the operator is. I envision that each one of the stages would be clearly marked(No. 1, No. 2, No. 3, etc.).

The purpose of inverting the cuvette assembly a few times is to mix the dilsim solution with the sample. Keep in mind the sample/dilsim solution is not dispensed into the Micro Titer well until the bar is "locked" into position and the assembly is inverted. The locking of the bar does two things (1) breaks the seal which houses the dilsim solution and (2) mixes the sample with the dilsim solution.

Step 4: Depress Bar until it "clicks" into position.

Depressing the bar until it "clicks" into position dispenses exactly 225ul of the dilsim/sample solution into the micro titer well. It is important to understand that accuracy is important but more important is the ability of the cuvette assembly to reproduce the volumes dispensed into the micro titer wells consistently.

Step 5: Place "Cuvette" assembly into infared warming tray for 90 to 100 minutes.

I envision the micro titer wells being the only thing that is heated. Therefore the tray itself would have a number of holes in which the bottom of the cuvette assembly would be inserted(the micro titer wells protruding out of the bottom of the cuvette assembly (approx 1/4" dia by 3/8" length).

Step 6: Remove "cuvette" assembly from Infared warming tray, move bar to was position (position No.2). Depress bar four times

This was station is hard for me to visualize since the details of the inner workings of this station were not detailed on the drawings. Note that each micro titer well would be washed simultaneously but on a mutual exclusive basis. In KEA's original design only one well was shown. Given that it is necessary to run a positive and negative control along with the patient sample(run in duplicate) four micro titer wells would be needed. Each of these wells would need to be washed at the same time.

Step 7: Move Bar to position No. 3 (labeled outside of Cuvette). Depress bar until it "clicks" into position.

The No. 3 position contains the Enzabody solution. Again the bar is utilized as a mechanical lever to dispense the 150ul of solution into the micro titer wells.

Step 8: Place "cuvette" assembly into Infared warming tray and incubate for 30 to 35 mins.

This step is the same as Step 5. Note that the temperature for incubation in steps 5 and 8 are the same.

Step 9: Remove "cuvette" assembly from Infared warming tray, move bar to wash position(position No.4). Depress bar four times

This is the same procedure as outlined in Step 6.

Step 10: Move bar to position No. 5. Depress bar until it "clicks into position.

Depressing the bar here dispenses the 150ul of substrate into the micro titer well.

Step 11: Place "cuvette" assembly into infared warming tray and incubate for 10 to 12 minutes

This incubation stage is different because of the difference in temperature. Note the 20 to 25 C here vs. the 37 C in steps 5 and 8.

Step 12: Remove "cuvette" assembly from infared warming tray and move bar to position No. 6. Depress bar until it "clicks" into position.

It is very important that the stopping solution be added simultaneously in all micro titer wells

The ability of the technician to reproduce this HIV-1 assay consistenly comes down to this step. The addition of the stopping solution is one of the weak points of a ELISA immunoassay.

Step 13: Read "cuvette" assembly within two hours.

ADDITIONAL COMMENTS:

In reviewing this protocol one of the concerns I have is the preparation of the solutions prior to mixing with the patient's sample. It was clearly outlined that the solutions should not be prepared prior to use more than 8 hours. It seems to me that this needs to be addressed.

SUBJECT: Report No. 3 from RAK Associates 10 January 1990

RE: Concerns regarding the cuvette design with reference to Organon Protocol.

REAGENT MIXING PRIOR TO TEST USE

Reference to the mixing of reagents prior to use: As was outlined in Organon Teknika's protocol, the various components ie. wash solution, dilsim, Enzabody, etc. required mixing (reconstituted) with the appropriate materials prior to use with the HIV-1 immunoassay. The shelf life after mixing was eight hours. Note that the mixing takes place at room temperature. I feel this area also needs to be addressed in the cuvette design.

If the wafer idea is put to work such that these premixed solution (dilsim, enzabody, wash) materials are encompassed in a waterproof plastic or glass contaainer, then the soluation can be placed in the appropriate stage of the cuvette and the material encapsulated in plastic or glass can be dropped and sealed in that stage also. When it comes time to mix these materials, the operator merely moves

the bar up(see earlier reports) to the appropriate stage, depress, and the wafer or glass capsul is broken. The operator merely inverts the cuvette assembly to cause mixing of this solution, wait a short period of time, then start the test according to the protocol.

STORAGE TEMPERATURE

Another area of concern is the storage of these materials at the 2 to 8 C as outlined in Organon's protocol. At this temperature materials are close to the freezing point. Therefore the material specification and design of the cuvette, including the wafer idea if used, must take into account the expansion and conatraction of fluids within this temperature range.

LABELLING

Bar Code labels are recommended on all products. The laboratory industry utilizes the standard codes. Each cuvette would need to have printed on it the Lot Number, date, manufacturer(Organon). I would also recommend that a large number appear on the top of the

cuvette so that the operator can identify which cuvette is being used for which patient. In addition, a place for a bar code label that the laboratory may want to affix to the cuvette may be in order.

SUBJECT: Report No. 4 from RAK Associates 5 February 1990

RE: Analysis of Monthly Technical Report No. 5

I. REAGENT ISSUES

A. Reagent Preparation

1. General: It appears that research is needed to ascertain the correct "internal" configuration of the various wafers required to be present within KEA's HIV cuvette. Although Organon's diagnostic kit is a model, the combination of reagents, dispensing times and amounts, as well as the temperature constraints are similar to other **ELISA** kits already on the market.

2. There are maybe up to seven wafers required or combination wafer/chambers for this cuvette. The objective of this discussion is not to identify the "chemical" means by which the immunoassay can be completed but to identify the mechanical mechanisms which optimize the existing methodology.

3. The Diluent: I envision a combination of a two chambered wafer combined with a well within the cuvette. By action of the operator the two chambered wafer is breeched with the (a) diluent concentrate and (b) between 20 materials mixing within the dionized water contained in the well. Temperature will be addressed later in this report.

4. The Positive and Negative Controls: I envision these being added by and operator via standard pipetting with an Eppendorf, MLA, SMI or other such pipetting device. The diluent which has been already prepared via 3. above would mix with the appropriate controls.

5. The Dilsim: I envision a single well in which the Dilsim is in a "dry powder" form. "Dry Powder" is not to be confused with a Lypholized type powder configuration. The objective here is to allow solubilization to occur quickly. Experimentation would be required as to the particle size, and amount of the "inert" filler required to prevent "caking" while maximizing "solubilization". It appears that a "three chambered" wafer

design may be in order. Temperature to be addressed later on in this report.

6. The Wash Solution(pages 24 & 27): fI envision a single well or single chambered wafer.

7. The Enzabody (page 25): This area presents some concern. I believe a "three" chambered wafer in cominiation with the well being filled with deionized water is in order. This would allow the Enzabody solution to be mixed just prior to use by the operator. I also recommend that a "dry powder" form for this protein be utilized in its preparation by the manufacturer vs a more standard "lypholized" method.

8. The ABTS Substrate (page 28): A single chambered wafer with the ABTS diluent filling the well. Temperature to be addressed later in this report.

9. The Stop Solution (page 30): A single chambered wafer of a type of material that is inert to the NaF solution.

B. **Temperature**

1. The storage temperature for this type of cuvette (2 to 8' C) preasents the problem of water condensation on the outside and inside of the cuvette when the cuvette is warmed to ambient temperature (20 to 25' C). As a result I would recommend that each cuvette be individually packaged in a nitrogen or other such inert atmosphere. The time in which the cuvette would be required to be warmed ie by leaving out on a laboratory bench would have to be determined. The danger I see if such percautions are not taken is further dilution of the various chemicals within the cuvette when the wafers are punctured. Keep in mind we are dealing with measurements within the 10-10 to 10-12 Molar range.

2. Experimentation is going to be required as to the different temperatures outlined in Organon's kit. In general if the cuvette can be kept at 37'C throughout the entire protocol, I believe all of the various reagents will reconstitute adequately and the end results should be sufficient. However, experimentation would need to be implemented to ascertain this assumption. I would

recommend that the temperature range from as low as 20'C to as high as 40'C be reviewed and correlated with the end results a known control for HIV.

C. **Time:**

1. Incubation times required would need to be varied and correlated with the various temperatures in (2). Keep in mind that immunoassays can give different results by varying the incubation temperature and time period. Further experimentation would be required here.

II. INSTRUMENTATION ISSUES

A. As discussed earlier, I believe the system should take into account two types of instrumentation hardware, (1) the incubation tray and (2) the spectrophotometer.

1. Incubation Tray: I envision a tray approximately 10" x 12" x 2" in size in which a number of cuvettes can be incubated simultaneously. There would be a red/green LED adjucent to each "cuvette nesting area"

which would be tied into a timer via some type of microprocessor. The idea would be that an operator merely drops the cuvette into the tray, push a button for that cuvette as to which part of the incubation cycle the cuvette is in and the tray would take care of the rest. The operator would be able to run a number of cuvettes in parallel at various stages within the immunoassay. This concept allows for a more "production line" type approach which realizes certain economics of scale for the operator. Obviously this tray would have some type of visual timing and temperature readout so that the operators could do the necessary "quality control" as required by many regulator agencies.

2. Spectrophotometer: I envision a simple, single chanbered, device with the appropriate data reduction capabiliaties to analyze the absorbance. This wouild be a separate instrument approximately 4" x 6" x 3". It is a single wavelength and is only used for "end point" determinations. Elisa methodologies are measured in an "end Point" determination.